Digestive and Liver Disease 51 (2019) 412–418

Contents lists available at ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Liver, Pancreas and Biliary Tract

Quantification of circulating miR-125b-5p predicts survival in chronic hepatitis B patients with acute-on-chronic liver failure

Ya-Chao Tao[a,b], Meng-Lan Wang[a,b], Ming Wang[a,b], Yuan-Ji Ma[a,b], Lang Bai[a,b], Ping Feng[a,b], En-Qiang Chen[a,b,*], Hong Tang[a,b,*]

[a] Center of Infectious Diseases, West China Hospital, Sichuan University, Chengdu, Sichuan, PR China
[b] Division of Infectious Diseases, State Key Laboratory of Biotherapy, Sichuan University, Chengdu, Sichuan, PR China

ARTICLE INFO

Article history:
Received 21 February 2018
Received in revised form 30 August 2018
Accepted 31 August 2018
Available online 11 September 2018

Keywords:
Acute-on-chronic liver failure
Chronic hepatitis B
MiR-125b-5p
Survival prediction

ABSTRACT

Aims: To analyze the role of serum miR-125b-5p in reflecting liver damage and predicting outcomes in chronic hepatitis B (CHB) patients with acute-on-chronic liver failure (ACLF).
Methods: CHB patients with normal hepatic function (n = 100), moderate-to-severe liver damage (n = 90), and ACLF (n = 136) were included. Among hepatitis B virus (HBV)-ACLF patients, 86 and 50 were in the training and validation cohorts, respectively. Serum miR-125b-5p level was measured by quantitative real-time PCR.
Results: Serum miR-125b-5p level increased with disease progression, and serum miR-125b-5p level was lower in surviving than in dead HBV-ACLF patients. Among HBV-ACLF patients, miR-125b-5p positively correlated with total bilirubin (TBil; $r = 0.214$, $p < 0.05$) and model for end-stage liver disease (MELD) score ($r = 0.382$, $p < 0.001$) and negatively correlated with prothrombin activity(PTA; $r = -0.215$, $p < 0.05$). MiR-122 showed a contrasting performance compared with miR-125b-5p. Cox regression analysis showed that miR-125b-5p, miR-122, and PTA were independent survival predictors for HBV-ACLF, and low miR-125b-5p and high miR-122 levels may predict a longer survival in HBV-ACLF. MiR-125b-5p (AUC = 0.814) had a higher performance for survival prediction in HBV-ACLF compared with miR-122 (AUC = 0.804), PTA (AUC = 0.762), MELD score (AUC = 0.799), and TBil (AUC = 0.670) alone; predictive effectiveness of miR-125b-5p was increased by combination with miR-122 (AUC = 0.898). MiR-125b-5p was an effective predictor of HBV-ACLF outcomes in the validation cohort.
Conclusions: MiR-125b-5p increase is associated with severity of liver damage; high serum miR-125b-5p may serve as a predictor for poor outcomes in HBV-ACLF cases.

1. Introduction

Acute-on-chronic liver failure (ACLF) is a life-threatening condition characterized by acute and rapid deterioration of previously well-compensated chronic liver diseases [1,2]. In China and most other Asian countries, hepatitis B virus (HBV)-associated ACLF (HBV-ACLF) patients account for more than 50% of ACLF patients, owing to the high prevalence of chronic HBV infection [3]; it has become one of the most lethal, prevalent, and cost-intensive diseases. In past decades, ACLF patients have often been reported to either undergo liver transplantation or die because of shortage of donor livers. One of the major obstacles in the treatment of ACLF is the lack of knowledge of the exact molecular pathogenesis and broad-spectrum specific treatments. Additionally, diagnostic delay or failure to diagnose contributes to the poor prognosis of HBV-ACLF [4,5]. Thus, it is of great importance to find new biomarkers for the early and accurate diagnosis of liver injury and disease progression in CHB patients.

MicroRNAs (miRNAs) are small, non-coding RNAs comprising 18–24 nucleotides, that regulate target gene or protein expression post-transcriptionally [6]. They play a role in various biological and pathological processes, including those in the liver [7]. Studies have demonstrated stable detection of miRNAs in the serum and plasma [8,9]. Because circulating miR-122 levels were first reported to predict drug-induced liver injury [10], increasing numbers of circulating miRNAs, such as miR-21 [11], miR-200a [12], and miR-223 [13], have been reported as biomarkers for liver injury. They may even be better biomarkers than classical parameters (such as

* Corresponding authors at: Center of Infectious Diseases, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, PR China.
E-mail addresses: chenenqiang1983@hotmail.com (E.-Q. Chen), htang6198@hotmail.com (H. Tang).

https://doi.org/10.1016/j.dld.2018.08.030
1590-8658/

ALT), as their levels fluctuate sooner than ALT levels during the course of liver damage [14]. Recently, circulating miRNAs (such as miR-125b-5p, miR-130a, miR-21-5p, and miR-143) have emerged as promising biomarkers for liver injury in CHB-related diseases. Among these circulating miRNAs, miR-125b-5p is of particular interest as miR-125b can regulate cell proliferation and apoptosis, and dysregulation of miR-125b has been found in several types of malignancies, including prostate cancer [15], non-small-cell lung cancer [16], and hepatocellular carcinoma (HCC) [17]. MiR-125b-5p, a member of miR-125b family, was recently reported to protect human hepatocytes against acute liver failure (ALF) [18]. However, its clinical validation data are still rare.

In this study, we analyzed the serum levels of circulating miR-125b-5p in CHB patients with varying degrees of liver damage and evaluated the predictive value of serum-circulating miR-125b-5p with regard to short-term mortality in cases of HBV-ACLF.

2. Patients and methods

2.1. Patients

This study was a post hoc measurement of prospectively collected blood samples (Registration number: ChiCTR-PRCH-13002984). All clinical information and serum samples of patients were obtained from the clinical biological sample library of our department from April 2016 to March 2018. In all, 326 CHB patients were finally enrolled, including 100 patients with normal hepatic function (Group A), 90 patients with moderate-to-severe liver damage (Group B), and 136 patients with ACLF. Among HBV-ACLF cases, 86 were used for the training model (Group C) and 50 (including 20 and 30 samples from surviving and dead patients, respectively) were assigned to the validation cohort to further identify and confirm our preliminary findings (Group D).

Patients were excluded if they were infected with other hepatitis viruses (including A, C, D, and E) or human immunodeficiency virus (HIV) or if they showed evidence of drug-induced liver injury, alcoholic liver disease (alcohol intake >40 g/d), autoimmune liver diseases, diabetes, severe systemic illnesses, HCC, and other tumors.

This study was conducted in accordance with clinical practice guidelines and was approved by the Ethics Committee for Human Experimentation at West China Hospital of Sichuan University.

2.2. Serum total RNA extraction and reverse transcription

Fresh blood was centrifuged at 3000 × g for 10 min at 4 °C, and the serum supernatant was collected, followed by additional centrifugation at 3000 × g at 4 °C for 10 min to remove any remaining cells. Subsequently, the aliquoted serum samples were immediately stored at −80 °C until further use. To verify the quality of analyzed serum, the collected samples were examined by visual inspection and spectrophotometric measurement at 414 nm to exclude hemolytic samples.

The isolation of serum miRNA was performed using 200 μL serum with miRcute miRNA extraction kit, according to the manufacturer's instructions (TIANGEN, China) [19]. The extracted RNA was reverse transcribed using the first-strand cDNA synthesis kit (Sangon Biotech, China), and the reverse transcription reaction was as follows: 10 μL miRNA RT Solution mix (2×), 2 μL miRNA RT Enzyme mix, 0.1 mg miRNA, and adding the reagent to 20 μL with nuclease-free water. Reaction conditions were 37 °C for 60 min, 85 °C for 5 min, and 4 °C until the end of the reaction.

2.3. Quantitative real-time RT-PCR

MiR-125b-5p and miR-122 expression was determined by quantitative real-time RT PCR using miRNA qPCR detection kit (Sangon Biotech, China), according to the manufacturer's instructions. The forward primer sequence for miR-125b-5p was CGTCCCTGAGACCCTAACTTGTGA, and forward primer sequence for miR-122 was CGTGGAGTGTGACAATGGTGTTTG. The reverse primers for miR-125b-5p or miR-122 were universal adaptor primers designed and provided by the Sangon Biotech Company (Shanghai, China). The reaction system was as follows: 10 μL miRNA qPCR master mix (2×), 0.5 μL forward primer, 0.5 μL reverse primer, 1.0 μL cDNA, and 8.0 μL of nuclease-free water. Reaction conditions were 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, and 60 °C for 30 min. All samples were measured in triplicates. MiR-125b-5p and miR-122 expression was normalized to endogenous control of U6, and the fold change was calculated using the $2^{-\Delta\Delta CT}$ method (Bio-Rad CFX Manager software).

2.4. Statistical analyses

Quantitative variables were expressed as the mean ± standard deviation (SD) or medians with interquartile ranges (IQRs), and categorical variables as absolute and relative frequencies. The *t*-test or nonparametric Mann–Whitney U-test or Kruskal–Wallis test was performed to calculated differences between quantitative data, as appropriate. Correlation between two quantitative variables was analyzed using spearman's bivariate correlation. The cut-off values for quantitative variables, including serum miR-125b-5p, serum miR-122, ALT, PTA, CHE, and MELD score, were calculated using receiver operating characteristic curve (ROC). These indicators were divided in two groups (high and low), according to their respective cut-off values, and then the survival predictors were determined using Cox's proportional hazards regression model. The area under the ROC curve was also used to evaluate the validity of miR-125b-5p for predicting the 28-day survival of patients. All statistical tests were two-sided and a *p*-value of less than 0.05 was considered to indicate a statistically significant difference. The statistical software package used was SPSS Version 18.0 for Windows (SPSS, Chicago, IL, USA).

3. Results

3.1. General characteristics of all patients

The detailed baseline characteristics of 276 patients with different severity of liver dysfunction are shown in Table 1; the main laboratory variables, such as prothrombin activity (PTA), total bilirubin (TBil), and MELD score, were significantly different among patients with normal hepatic function (Group A), moderate-to-severe liver damage (Group B), and ACLF (Group C). The clinical characteristics of Group D (validation cohort) were similar to those of group C and no significant differences were found between them (Table 1). Among HBV-ACLF patients in Group C (Table 2), the values for PTA, TBil, creatinine (Cr), cholinesterase (CHE), and MELD score were all significantly different between surviving and dead patients ($p < 0.05$).

3.2. Serum miR-125b-5p expression in patients with different severity of liver damage

As shown in Fig. 1, serum levels of miR-125b-5p and miR-122 varied among three groups. Compared with the endogenous control, the mean fold change of serum miR-125b-5p was 1.38 ± 0.48, 1.83 ± 0.82, and 3.06 ± 1.20 for patients in Groups A, B, and C, respectively ($p < 0.01$ for all comparisons; Fig. 1A). This finding

Table 1
Clinical characteristics of all patients according to severity of liver damage.

Variables	Group A (N = 100)	Group B (N = 90)	Group C (N = 86)	Group D (N = 50)	*p* Value		
					Group B *vs.* A	Group C *vs.* B	Group C *vs.* D
Age, years	43.2 ± 11.0	42.1 ± 10.9	44.7 ± 10.4	45.0 ± 9.8	0.025	0.001	0.967
Gender, M/F	76/24	67/23	74/12	40/10	0.806	0.054	0.356
PTA, %	137.6 (113.3–164.1)	74.2 ± 11.9	33.9 ± 11.8	30.2 ± 11.5	0.001	0.000	0.459
TBil, μmol/L	16.0 (10.0–30.0)	116.5 (55.6–189.0)	357.5 (250.9–460.4)	385.5 (250.9–497.2)	0.001	0.001	0.755
ALT, ULN (IU/L)	26.0 (17.2–39.8)	328.5 (131.0–898.0)	264.0 (104.8–493.8)	255.5 (127.5–423.3)	0.001	0.113	0.896
ALB, g/L	49.5 (44.5–53.2)	33.8 (30.1–41.0)	31.6 (29.5–34.1)	31.5 (28.6–34.2)	0.001	0.002	0.839
Amon, mol/L	19.0 (13.0–24.0)	56.5 (45.0–72.0)	66.0 (50.0–93.0)	68.0 (45.8–95.0)	0.001	0.012	0.761
Cr, μmol/L	69.0 (63.0–78.0)	83.4 (69.0–95.6)	78.5 (60.0–101.0)	81.5 (56.5–134.0)	0.001	0.399	0.610
CHE, $\log_{10}$ IU/L	3.97 (3.84–3.97)	3.7 (3.7–3.8)	3.6 (3.5–3.7)	3.6 (3.4–3.7)	0.001	0.001	0.068
WBC, $\times 10^9$/L	7.03 (5.84–8.33)	5.3 (4.3–6.8)	6.5 (5.60–8.87)	6.78 (5.54–9.09)	0.001	0.001	0.623
HBeAg (+/−)	64/36	55/35	30/56	21/29	0.681	0.001	0.408
HBV DNA, $\log_{10}$IU/mL	5.33 (3.93–6.84)	4.61 (3.00–6.31)	4.39 (3.00–6.33)	4.77 (3.66–5.67)	0.045	0.285	0.509
Cirrhosis-n%	0	20 (21.7%)	76 (88.4%)	44(88.0%)	0.000	0.000	0.948
MELD score	NG	15.0 (10.6–17.9)	24.2 (20.4–28.6)	24.6 (19.2–35.4)	NG	0.001	0.591

Abbreviations: PTA: prothrombin activity; TBil: total bilirubin; Amon: ammonia; Cr: creatinine; CHE: cholinesterase. Group C was the ACLF training model cohort, while Group D was the ACLF validation cohort.

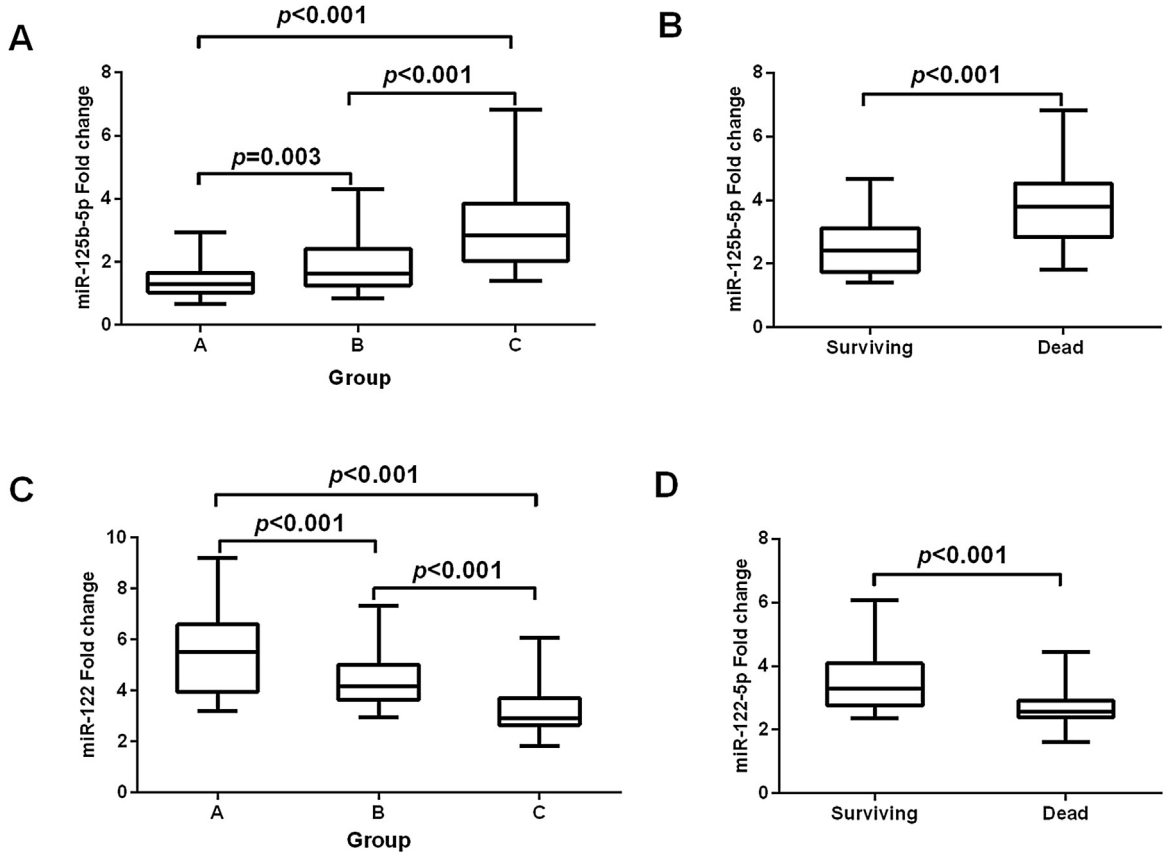

Fig. 1. Serum levels of miR-125b-5p (A,B) and miR-122 (C,D) in patients with normal hepatic function (N = 100, Group A), moderate-to-severe liver damage (N = 90, Group B), and ACLF(N = 86, Group C) in the training cohort. The analysis shows that the two miRNA expressions are significantly different among the three groups (A,C) and between surviving and death among HBV-ACLF patients (B,D). The vertical lines indicate the range, and the horizontal boundaries of the boxes represent the first and the third quartile.

suggested that serum miR-125b-5p levels significantly increased with disease progression. Among HBV-ACLF patients in the training cohort (Group C), the mean fold change of serum miR-125b-5p was significantly lower in surviving than in dead patients(2.52 ± 0.83 *vs.* 3.82 ± 1.23; $p < 0.001$; Fig. 1B).

In contrast, serum miR-122 levels decreased with disease progression. Compared with the endogenous control, the mean fold change of serum miR-122 was 5.48 ± 1.68, 4.46 ± 1.05, and 3.27 ± 0.91 for patients in Group A, B, and C, respectively ($p < 0.001$ for all comparisons; Fig. 1C). Among HBV-ACLF patients in the training cohort (Group C), mean fold change of serum miR-122 was significantly higher in surviving than in dead cases (3.60 ± 0.99 *vs.* 2.61 ± 0.53; $p < 0.001$; Fig. 1D).

3.3. Correlation between serum miR-125b-5p levels in the training cohort and general clinical characteristics

Because PTA and TBil are two important factors for ACLF diagnosis, and MELD score is associated with the severity and prognosis of ACLF, it is necessary to analyze the correlation between serum miR-125b-5p and PTA, TBil, and MELD score. Serum miR-125b-5p level was found to positively correlate with TBil (r = 0.214, $p < 0.05$) and

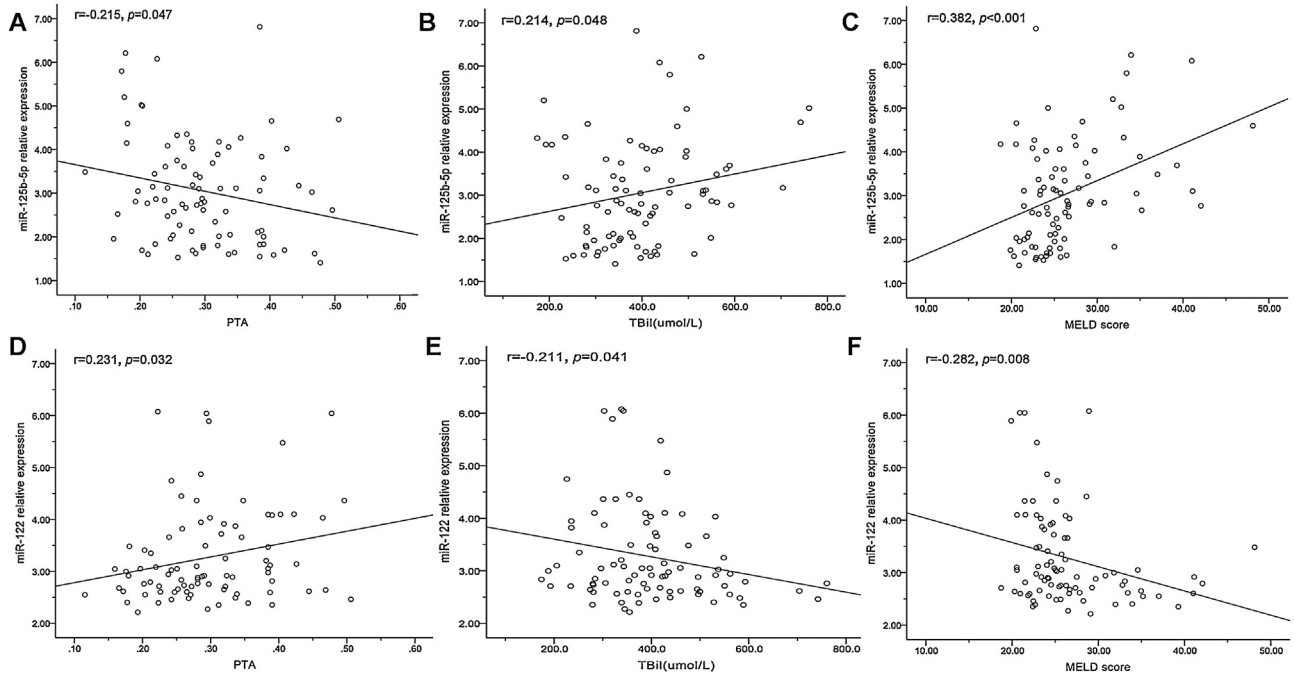

Fig. 2. Correlation of serum miR-125b-5p in the training cohort with PTA, TBil, and MELD score (A–C), and the correlation of serum miR-122 with the three clinical characteristics (D–F).

Table 2
Clinical characteristics of ACLF patients with different outcomes in the training cohort.

Variables	Surviving (N = 50)	Dead (N = 36)	*p*-Value
Age, years	44.3 ± 11.2	45.3 ± 9.2	0.668
Gender, M/F	42/8	32/4	0.514
PTA, %	36.2 ± 11.2	26.5 ± 11.0	0.001
TBil, μmol/L	327.8 (237.4–426.5)	422.6 (322.5–531.9)	0.024
ALT, ULN (IU/L)	262.5 (125.5–467.3)	271.0 (81.8–572.8)	0.652
ALB, g/L	32.5 ± 4.3	31.1 ± 4.0	0.107
Amon, mol/L	70.0 (49.3–90.3)	68.0 (49.5–102.5)	0.684
Cr, μmol/L	76.0 (61.5–85.3)	103.5 (62.0–215.0)	0.005
CHE, $\log_{10}$ U/L	3.3 ± 0.16	3.5 ± 0.16	0.003
WBC, $\times 10^9$/L	6.4 (5.4–8.1)	6.78 (5.6–11.8)	0.179
HBeAg (+/−)	20/30	10/26	0.241
HBV DNA	4.36 (3.00–6.54)	4.51 (3.00–6.12)	0.493
Cirrhosis-n%	42 (84%)	34 (94.4%)	0.136
MELD score	22.1 ± 3.7	29.2 ± 9.0	0.001

MELD score (r = 0.382, $p < 0.01$), but negatively correlate with PTA (r = −0.215, $p < 0.05$). In contrast, serum miR-122 level positively correlated with PTA (r = 0.231, $p < 0.05$), but negatively correlated with TBil (r = −0.221, $p < 0.05$) and MELD score (r = −0.282, $p < 0.01$). However, the correlations were relatively weak for both serum miR-125b-5p and miR-122 levels with PTA, TBil, and MELD score (Fig. 2).

3.4. Circulating miR-125b-5p predicting the 28-day survival of HBV-ACLF patients in the training cohort

The univariate and multivariate analysis results are shown in Table 3. As presented in the univariate analysis, serum miR-125b-5p, miR-122, PTA, TBil, ALB, blood ammonia, Cr, CHE, and MELD score were significantly different between surviving and dead patients; however, there were no statistically significant differences in sex, age, ALT levels, and white blood cell count between surviving and dead ACLF patients. In multivariate Cox regression analysis, serum miR-125b-5p, miR-122, PTA, and CHE were significant independent predictors for the overall survival of HBV-ACLF patients.

The AUC values of clinical variables for predicting the overall survival of HBV-ACLF patients are shown in Fig. 3. The AUC value was 0.814 for miR-125b-5p (95%CI: 0.715–0.889), 0.804 for miR-122 (95% CI: 0.704–0.882), 0.762 for PTA (95% CI: 0.659–0.848), 0.670 for TBil (95% CI: 0.560–0.768), and 0.799 for MELD score (95% CI: 0.699–0.878). Although serum miR-125b-5p showed a higher performance for survival prediction in HBV-ACLF compared with serum miR-122, PTA, TBil, and MELD score alone, the difference in AUC values between serum miR-125b-5p and the other four variables [serum miR-122($p = 0.888$), PTA ($p = 0.498$), TBil ($p = 0.048$) and MELD score ($p = 0.829$)] was not always significantly. However, the AUC of miR-125b-5p + miR-122 combined was 0.898 (95% CI: 0.814–0.953) for predicting overall survival in HBV-ACLF, which was significantly higher than that of either miR-125b-5p ($p = 0.02$) or miR-122 ($p = 0.027$) alone (Fig. 3C). Thus, to a certain extent, the predictive effectiveness of serum miR-125b-5p was greatly increased by the combination with serum miR-122.

As mentioned above, univariate Cox regression analysis revealed significant associations of serum miR-125b-5p ($p < 0.001$; HR: 5.341; 95% CI: 2.709–10.531) and serum miR-122 ($p < 0.001$; HR: 0.103; 95% CI: 0.031–0.336) with overall survival of HBV-ACLF patients (Table 3). Therefore, HBV-ACLF patients were divided into low and high serum miR-125b-5p or miR-122 groups, according to the cut-off value of serum miRNA concentration (3.464 for serum miR-125b-5p and 3.129 for serum miR-122). As per the survival curves shown in Fig. 3, low serum levels of miR-125b-5p (Fig. 3D) and high serum levels of miR-122 (Fig. 3E) were associated with longer survival of HBV-ACLF patients.

3.5. The verification of serum miR-125b-5p levels in predicting HBV-ACLF outcome in validation cohort

We verified preliminary findings with 50 ACLF serums samples from the validation cohort and found that the mean fold change of serum miR-125b-5p in surviving patients was significantly lower than that in dead cases (2.36 ± 0.63 *vs.* 3.72 ± 1.34, $p < 0.001$; Sup-

Table 3
Univariate and multivariate analyses of parameters associated with overall survival of HBV-ACLF patients in the training cohort.

Parameter	No. of patients (%)	Univariate regression analysis			Multivariate regression analysis		
		HR	95%CI	*p* Value	HR	95%CI	*p* Value
Serum miR-125b-5p							
Low miR-125b-5p	59 (68.6)	Ref					
High miR-125b-5p	27 (31.4)	5.341	2.709–10.531	<0.001	2.788	1.353–5.746	0.005
Serum miR-122							
Low miR-122	54 (62.8)	Ref					
High miR-122	32 (37.2)	0.103	0.031–0.336	<0.001	0.187	0.053–0.659	0.009
Age, years							
<60	78 (90.7)	Ref					
≥60	8 (9.3)	0.863	0.265–2.817	0.808			
Sex							
Male	74 (86.0)	Ref					
Female	12 (24.0)	0.791	0.280–2.237	0.658			
ALT, IU/L							
<698	75 (87.2)	Ref					
≥698	11 (12.8)	2.104	0.919–4.814	0.078			
PTA							
<0.271	35 (40.7)	Ref					
≥0.271	51 (59.3)	0.239	0.119–0.480	<0.001	0.386	0.182–0.820	0.013
TBil, μmol/L							
<436.5	61 (70.9)	Ref					
≥436.5	25 (29.1)	3.074	1.593–5.934	0.001	1.592	0.782–3.244	0.200
ALB, g/L							
<33	55 (64.0)	Ref					
≥33	31 (36.0)	0.414	0.189–0.910	0.028	0.604	0.253–1.447	0.258
Amon, mol/L							
<97.5	67 (77.9)	Ref					
≥97.5	19 (22.1)	2.232	1.112–4.482	0.024	1.499	0.707–3.179	0.291
Cr, μmol/L							
<101.5	64 (74.4)	Ref					
≥101.5	22 (25.6)	4.904	2.527–9.517	<0.001	0.732	0.255–2.104	0.562
CHE, IU/L							
<4500	56 (65.1)	Ref					
≥4500	30 (34.9)	0.234	0.091–0.602	0.003	0.246	0.081–0.744	0.013
WBC, $\times 10^9$/L							
<4	15 (17.4)	Ref					
4–10	58 (67.5)	0.975	0.393–2.417	0.957			
≥10	13 (15.1)	2.462	0.874–6.934	0.088			
MELD score							
<26.5	57 (66.3)	Ref					
≥26.5	29 (33.7)	7.290	3.591–14.802	<0.001	2.736	0.862–8.687	0.088

Abbreviations: HR, hazard radio; CI, confidence interval. Serum miR-125b-5p, miR-122, ALT, PTA, TBil, ALB, Amon, Cr, CHE and MELD score were divided into high and low groups in terms of their respective cut-off values.

plemental Fig. S1A in the online version at DOI: 10.1016/j.dld.2018.08.030). Serum miR-125b-5p seemed superior to TBil for predicting the survival of HBV-ACLF patients ($p = 0.034$), whereas no significant difference was observed between the AUC of serum miR-125b-5p and that of other the two clinical variables [PTA ($p = 0.700$) and MELD score ($p = 0.804$)], quite consistent with the preliminary studies (Supplemental Fig. S1B in the online version at DOI: 10.1016/j.dld.2018.08.030).

4. Discussion

Currently, no satisfactory indicators have been well and successfully applied to assess HBV-ACLF progression and prognosis. Therefore, an accurate assessment of disease progression and an early biomarker for prognosis is of great importance. The miRNA research has been a hotspot in recent years, and miR-125b has proved to play an important role in HCC [17], HBV replication [20], and ALF [18]. In this study, we investigated the association of serum miR-125b-5p with liver damage severity and prognosis of patients with HBV-ACLF in a real-world cohort. Before assessing the relative expression of miRNA, we evaluated level of hemolysis in all samples by visual inspection and absorbance measurement at 414 nm to eliminate defective samples for accurate quantification of miRNA in the serum [21]. The major findings with regard to serum miR-125b-5p in the present study were as follows: (1) serum miR-125b-5p levels significantly increased with increasing severity of liver damage and serum miR-125b-5p levels were lower in surviving than in dead HBV-ACLF patients; (2) serum miR-125b-5p positively correlated with TBil and MELD score and negatively correlated with PTA among HBV-ACLF patients, but the correlations were all relatively weak; (3) serum miR-125b-5p was an independent predictor of overall survival in HBV-ACLF cases, with higher performance compared with serum miR-122, PTA, TBil, and MELD score alone.

Serum miRNAs have recently been found to be involved in liver injury [22,23]. In this study, serum miR-125b-5p varied along with disease progression. Compared with patients with normal liver function, serum miR-125b-5p showed a gradually increasing trend among patients with moderate-to-severe liver damage and those with HBV-ACLF. Additionally, we found that serum miR-125b-5p level was higher in all three groups than in healthy volunteers (data not shown). In fact, a recently published study reported that CHB patients with moderate-to-severe liver necroinflammation ($G \geq 2$) had higher serum miR-125b levels than those with mild liver necroinflammation ($G < 2$) [20], which was basically consistent with the present findings. We also compared miR-125b-5p expression in different HBeAg statuses but found no significant differences. However, a significant difference with regard to serum miR-125b-5p was observed between cirrhotic and non-cirrhotic patients ($p < 0.01$, data not shown). In this study, we observed that

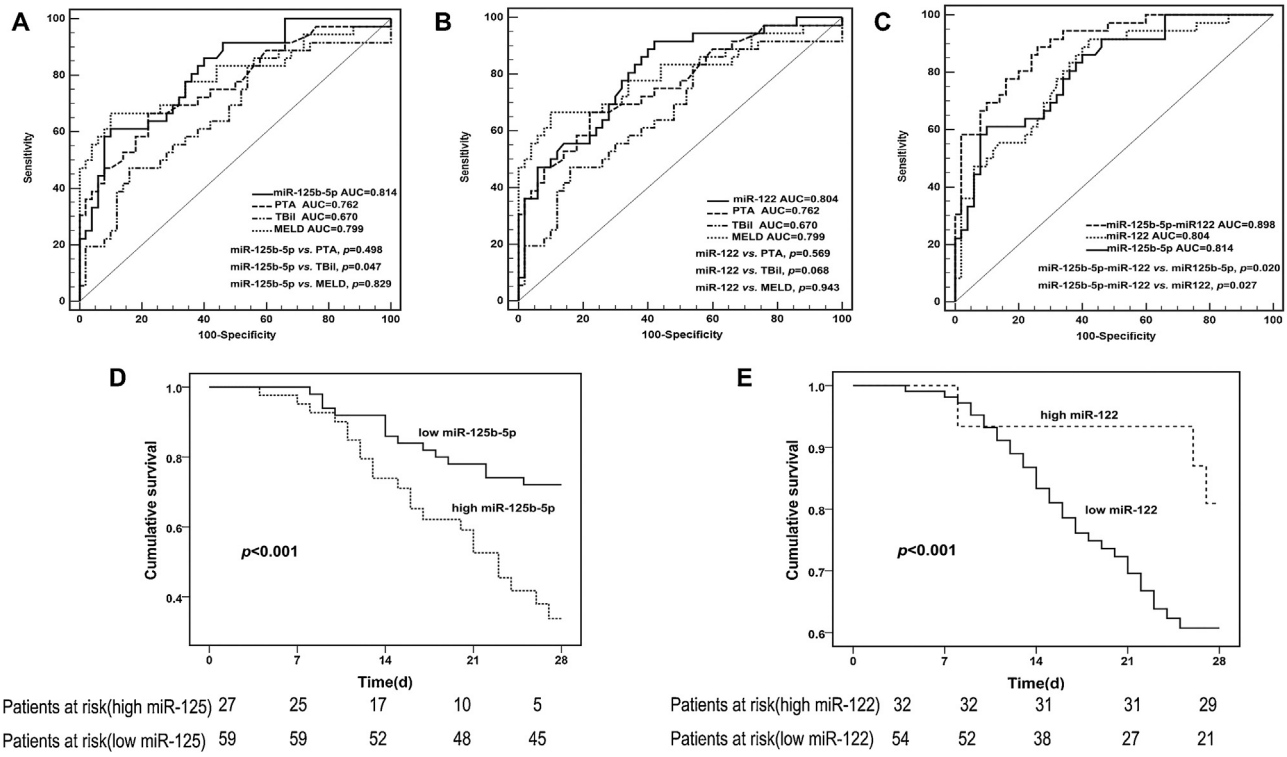

Fig. 3. The AUC comparison of serum miR-125b-5p (A) and miR-122 (B) detected in the training cohort with PTA, TBil, and MELD score and the AUC comparison of combined miRNAs with miR-125b-5p or miR-122 alone (C). The 28-day survival curves for HBV-ACLF patients with low or high serum miR-125b-5p (D) or miR-122 (E) levels. The *p* values were calculated with the Cox regression model.

miR-125b-5p expression was lower in surviving than that in dead patients both in the training and validation ACLF cohorts, and this finding was in agreement with the previous finding in ALF that miR-125b-5p expression was lower in the sera of patients who spontaneously recovered than in the sera of those who did not [18].Thus, dynamic monitoring the serum levels of miR-125b-5p would help early prediction for further deterioration of liver function.

Intriguingly, serum miR-122 showed a contrasting performance compared with serum miR-125b-5p. Its levels were downregulated as the liver injury severity increased and higher serum miR-122 levels were found in CHB patients with mild liver damage than in those with severe liver damage and liver failure; similarly, higher levels were found in surviving than in dead ACLF patients. These results were in agreement with other similar studies [24–26]. Indeed, high levels of serum miR-122 in surviving individuals were also observed in patients with ALF [24] and liver cirrhosis [26], which to some extent was consistent with the present result. We had to mention that there seemed to be a negative correlation between miR-125b-5p in peripheral blood and liver tissues because of an overall declining trend of miR-125b-5p in liver tissue with increasing severity of liver damage (data not shown). We speculated that miR-125b-5p originates from hepatocytes or other liver cells in the form of exosomes, microvesicles, or apoptotic bodies [27]. It may be released into circulation when the liver exposes to stress or injury. The presence of miR-125b-5p in the serum is thought to correlate with liver damage, and could reflect the severity of liver injury to some extent. However, it is impossible to always maintain a high level of serum miR-125b-5p during the whole course of ACLF. It is well known that liver regeneration plays a key role in the process of ACLF [28]. Thus, if the number of regenerative cells exceeds that of apoptotic cells or if a patient is at a very late stage of ACLF, we speculate that the miR-125b-5p in the peripheral blood and liver tissues both should be decreased.

As reported in this study serum miR-122 showed a completely opposite performance compared with serum miR-125b-5p. However, the mechanisms of contrasting performances of serum miR-125b-5p and miR-122 remain obscure. Based on the existing limited data, the different expression levels of miRNAs in the serum may be relevant in the selective packaging of exosomes [29]. Moreover, miR-125b-5p and miR-122 have a different effect on HBV replication. Evidence has shown that miR-125b-5p could stimulate HBV replication through its target LIN28B/let-7 axis, whereas miR-122 could inhibit HBV replication by down-regulating cyclin G1 and blocking the interaction of between cyclin G1 and p53 [30]. Thus, the contrasting performances of serum miR-125b-5p and miR-122 may indicate a complex interaction network among miRNAs, viral replication, and liver damage.

Recently, circulating miR-125b-5p was also considered an indicator of acetaminophen (APAP)-induced liver damage and its levels were dramatically elevated before ALT, rising to above 1000 U/L [14]. Moreover, miR-125b-5p could distinguish APAP-induced liver damage from ischemic-hepatitis damage, suggesting that miR-125b-5p may have an advantage over ALT and some other common clinical indicators in reflecting liver damage. In the present study, we observed that serum miR-125b-5p weakly correlated with clinical parameters involved in ACLF, such as PTA, TBil, and MELD score; AUC value for serum miR-125-5p was also higher than that for PTA, TBil, MELD score, and miR-122. Importantly, the predictive effectiveness of serum miR-125b-5p in HBV-ACLF was greatly increased by combination with serum miR-122, with high sensitivity and specificity. Thus, quantitative measurement of serum miR-125-5p may serve as a new important tool for the management of HBV-ACLF.

There were some limitations in this study. First, healthy volunteers should be included to confirm the normal levels of serum miR-125b-5p. Second, miRNA coming from tissues and exosomes should also be extracted and analyzed, as it would help give a more

comprehensive assessment of miRNA change in deterioration of liver function. Third, this was a single-center study with a relatively small sample size. Thus, large multicenter cohort studies are required to confirm present findings.

In conclusion, serum circulating miR-125b-5p varies with the deterioration of liver function. The increase serum circulating miR-125b-5p is associated with the severity of liver damage, and high serum miR-125b-5p level may serve as a novel predictor for poor outcome in HBV-ACLF. However, because of the complicated performance of miRNA in cases of viral replication [20,30], HBeAg status [31], and liver damage, more studies are needed to fully reveal the molecular mechanism of miR-125b-5p involved in the pathophysiological process and the outcome in HBV-ACLF cases.

Conflict of interest

None declared.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (81770620) and Science and Technology Support Program of Sichuan Province, China (2016SZ0042 and 2015SZ0049). We appreciate the assistance from Dong-Mei Zhang with regard to patient follow-up and Min He and Cong Liu with regard to collection and separation of blood samples.

References

[1] Zamora Nava LE, Aguirre Valadez J, Chavez-Tapia NC, Torre A. Acute-on-chronic liver failure: a review. Ther Clin Risk Manag 2014;10:295–303.
[2] Asrani SK, Simonetto DA, Kamath PS. Acute-on-chronic liver failure. Clin Gastroenterol Hepatol 2015;13:2128–39.
[3] Sarin SK, Kedarisetty CK, Abbas Z, Amarapurkar D, Bihari C, Chan AC, et al. Acute-on-chronic liver failure: consensus recommendations of the Asian Pacific Association for the Study of the Liver (APASL) 2014. Hepatol Int 2014;8:453–71.
[4] Chan HL. Antiviral therapy for acute-on-chronic liver failure in chronic hepatitis B: is it too late? Dig Dis Sci 2010;55:2126–8.
[5] Graziadei IW. The clinical challenges of acute on chronic liver failure. Liver Int 2011;31(Suppl. 3):24–6.
[6] O'Connell RM, Rao DS, Baltimore D. microRNA regulation of inflammatory responses. Annu Rev Immunol 2012;30:295–312.
[7] Szabo G, Bala S. MicroRNAs in liver disease. Nat Rev Gastroenterol Hepatol 2013;10:542–52.
[8] Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008;105:10513–8.
[9] Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 2008;18:997–1006.
[10] Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proc Natl Acad Sci U S A 2009;106:4402–7.
[11] Watany MM, Hagag RY, Okda HI. Circulating miR-21, miR-210 and miR-146a as potential biomarkers to differentiate acute tubular necrosis from hepatorenal syndrome in patients with liver cirrhosis: a pilot study. Clin Chem Lab Med 2018;56:739–47.
[12] Austermann C, Schierwagen R, Mohr R, Anadol E, Klein S, Pohlmann A, et al. microRNA-200a: a stage-dependent biomarker and predictor of steatosis and liver cell injury in human immunodeficiency virus patients. Hepatol Commun 2017;1:36–45.
[13] Schueller F, Roy S, Loosen SH, Alder J, Koppe C, Schneider AT, et al. miR-223 represents a biomarker in acute and chronic liver injury. Clin Sci (Lond) 2017;131:1971–87.
[14] Ward J, Kanchagar C, Veksler-Lublinsky I, Lee RC, McGill MR, Jaeschke H, et al. Circulating microRNA profiles in human patients with acetaminophen hepatotoxicity or ischemic hepatitis. Proc Natl Acad Sci U S A 2014;111:12169–74.
[15] Shi XB, Xue LR, Ma AH, Tepper CG, Kung HJ, White RWD. miR-125b promotes growth of prostate cancer xenograft tumor through targeting pro-apoptotic genes. Prostate 2011;71:538–49.
[16] Li QC, Han Y, Wang CH, Shan S, Wang YY, Zhang JG, et al. MicroRNA-125b promotes tumor metastasis through targeting tumor protein 53-induced nuclear protein 1 in patients with non-small-cell lung cancer. Cancer Cell Int 2015:15.
[17] Chen SS, Chen H, Gao SS, Qiu SL, Zhou H, Yu MX, et al. Differential expression of plasma microRNA-125b in hepatitis B virus-related liver diseases and diagnostic potential for hepatitis B virus-induced hepatocellular carcinoma. Hepatol Res 2017;47:312–20.
[18] Yang D, Yuan Q, Balakrishnan A, Bantel H, Klusmann JH, Manns MP, et al. MicroRNA-125b-5p mimic inhibits acute liver failure. Nat Commun 2016;7:11916.
[19] Wang JY, Mao RC, Zhang YM, Zhang YJ, Liu HY, Qin YL, et al. Serum microRNA-124 is a novel biomarker for liver necroinflammation in patients with chronic hepatitis B virus infection. J Viral Hepat 2015;22:128–36.
[20] Li F, Zhou P, Deng W, Wang J, Mao R, Zhang Y, et al. Serum microRNA-125b correlates with hepatitis B viral replication and liver necroinflammation. Clin Microbiol Infect 2016;22:384, e1-e10.
[21] Kirschner MB, Kao SC, Edelman JJ, Armstrong NJ, Vallely MP, van Zandwijk N, et al. Haemolysis during sample preparation alters microRNA content of plasma. PLoS One 2011;6:e24145.
[22] Yang R, Yang F, Huang ZL, Jin Y, Sheng YC, Ji LL. Serum microRNA-122-3p, microRNA-194-5p and microRNA-5099 are potential toxicological biomarkers for the hepatotoxicity induced by Airpotato yam. Toxicol Lett 2017;280:125–32.
[23] Howell LS, Ireland L, Park BK, Goldring CE. MiR-122 and other microRNAs as potential circulating biomarkers of drug-induced liver injury. Expert Rev Mol Diagn 2018;18:47–54.
[24] John K, Hadem J, Krech T, Wahl K, Manns MP, Dooley S, et al. MicroRNAs play a role in spontaneous recovery from acute liver failure. Hepatology 2014;60:1346–55.
[25] Waidmann O, Bihrer V, Pleli T, Farnik N, Berger A, Zeuzem S, et al. Serum microRNA-122 levels in different groups of patients with chronic hepatitis B virus infection. J Viral Hepat 2012;19:e58–65.
[26] Waidmann O, Koberle V, Brunner F, Zeuzem S, Piiper A, Kronenberger B. Serum microRNA-122 predicts survival in patients with liver cirrhosis. PLoS One 2012;7:e45652.
[27] Yang X, Weng ZQ, Mendrick DL, Shi Q. Circulating extracellular vesicles as a potential source of new biomarkers of drug-induced liver injury. Toxicol Lett 2014;225:401–6.
[28] Karp SJ. Biology of hepatocyte regeneration in acute liver failure. Liver Transpl 2015;21:S34–5.
[29] Holman NS, Mosedale M, Wolf KK, LeCluyse EL, Watkins PB. Subtoxic alterations in hepatocyte-derived exosomes: an early step in drug-induced liver injury? Toxicol Sci 2016;151:365–75.
[30] Deng W, Zhang X, Ma Z, Lin Y, Lu M. MicroRNA-125b-5p mediates post-transcriptional regulation of hepatitis B virus replication via the LIN28B/let-7 axis. RNA Biol 2017;14:1389–98.
[31] Giray BG, Emekdas G, Tezcan S, Ulger M, Serin MS, Sezqin O, et al. Profiles of serum microRNAs; miR-125b-5p and miR223-3p serve as novel biomarkers for HBV-positive hepatocellular carcinoma. Mol Biol Rep 2014;41:4513–9.